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Mice with the testicular feminization mutation demonstrate a role for androgen receptors in the regulation of anxiety-related behaviors and the hypothalamic–pituitary–adrenal axis

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ABSTRACT

Testosterone (T) appears to play a role in anxiety and sensorimotor gating in rodents, but whether T acts through the androgen receptor (AR) to influence these behaviors is less clear. We compared adult genetic male mice with the testicular feminization mutation (Tfm), which lack functional ARs, to wild type male littermates (wt males) on an assay of sensorimotor gating (prepulse inhibition of the acoustic startle response; PPI) and several tests thought to reflect anxiety: open field exposure, novel object exposure, elevated plus maze (EPM), and light/dark (LD) box. PPI was similar between groups, but indices of anxiety in the novel object and LD box tests were increased in Tfm males with no significant differences found in the open field or EPM. Since Tfm male mice have decreased circulating T, the same tests were conducted in mice that were gonadectomized (wt males) or sham-operated (Tfm males) as adults and supplemented with T or nothing (B). While T treatment reduced indices of anxiety in the novel object and LD box tests in wt males, it was ineffective in Tfm males. Increased indices of anxiety in Tfm males appear to be related to hyper-activation of the hypothalamic–pituitary–adrenal axis since levels of the stress hormone corticosterone were elevated in Tfm males compared to wt males at baseline and at several time points after exposure to a novel object. These findings demonstrate that ARs influence anxiety and stress responses in mice.

Introduction

In humans, gonadal hormones have been implicated in the development and maintenance of several mental disorders including anxiety, depression, and schizophrenia. Women suffer from anxiety disorders and depression more frequently than do men (Weinstock, 1999; Kornstein, 1997; Wilhelm et al., 1998), and variations in the ovarian hormone estrogen appear to contribute to the etiology of these disorders in women (Arpels, 1996; Yazici et al., 2003). Evidence in animal models supports a role of estrogen in mood disorders. In both male and female rodents, estrogens have been shown to decrease depressive and anxiety-related behavior (Frye and Lacey, 2001; Walf and Frye, 2005), particularly through activation of the estrogen receptor (ER) isoform ERβ (Lund et al., 2005; Imwalle et al., 2005).

Emerging evidence suggests that androgens also contribute to mood disorders. Boys and girls with low T levels show increased indices of depression and anxiety compared to those with high T (Granger et al., 2003). Furthermore, in hypogonadal and aging men who have low levels of circulating T, mood disorders are increased (Kaminetsky, 2005; Lund et al., 1999; Amore, 2005; Eskelinen et al., 2007; Cooper and Ritchie, 2000). T treatment of these individuals can decrease symptoms of depression and anxiety (Kumano, 2007; Kaminetsky, 2005; Cooper and Ritchie, 2000).

Androgens have also been shown to play a role in anxiety-related behavior in rodents, with an increase in T generally correlated with decreased indices of anxiety (Frye et al., 2008; Bing et al., 1998; Bitran et al., 1993). Reducing androgen levels via gonadectomy in male rodents increases anxiety and fear-related behaviors (Adler et al., 1999; Bitran et al., 1993; Frye et al., 2008; Frye and Edinger, 2004). Supplementation with T also decreases indices of anxiety in several rodent models (Frye et al., 2008; Aikey et al., 2002; Edinger and Frye, 2005). As in hypogonadal men, diminished testicular production of T in mice is associated with anxiogenesis and depressive behaviors (Umehara et al., 2006). In rodents, aging, which has also been correlated with increased anxiety-related behaviors (Koprowska et al., 2004; Boguszewski and Zagrodzka, 2002), is accompanied by a decline in T. A recent study suggests that anxiety-related behavior in aged male mice can be reduced by administration of androgens (Frye et al., 2008).

One mechanism through which androgens may influence anxiety-related behavior is through the regulation of the hypothalamic–pituitary–adrenal (HPA) axis. While HPA axis activation is generally adaptive, hyper-activation may be maladaptive and has been associated with increased indices of anxiety and depression in both humans and rodents (Scott and Dinan, 1998; Landgraf et al., 1999;

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Lund et al., 2005). In rodents, administration of T, and its metabolites dihydrotestosterone (DHT) and 5alpha-androstane, 3beta,17beta-diol (3β-diol), can suppress the normal rise of stress hormones (adrenocorticotropic hormone (ACTH) from the pituitary and corticosterone from the adrenal cortex) following exposure to a stressful situation (Handa et al., 1994; Lund et al., 2004; Lund et al., 2005; Lund et al., 2006), suggesting androgens can influence HPA axis activity, and in turn, may potentially influence anxiety-related behaviors.

Gonadal hormones, particularly estrogens, have also been hypothesized to play a neuroprotective role in schizophrenia, which may contribute to the later onset and milder symptoms of this disease in women compared to men (Häfner et al., 1998; Castle et al., 1995). One common characteristic in people with schizophrenia is a deficit in sensorimotor gating, the capacity to filter sensory, motor, and cognitive information (Kodsi and Swerdlow, 1994). This reduction in sensorimotor gating may be linked to symptoms of schizophrenia such as information processing deficits, disorders of thought and auditory hallucinations (Perry and Braff, 1994; Kumari et al., 2008). In an operational measure of sensorimotor gating, prepulse inhibition of the acoustic startle response (PPI; Swerdlow et al., 1996), people with schizophrenia show reduced PPI indicating a deficit in sensorimotor gating (Braff et al., 1992). This deficit is alleviated by treatment with atypical antipsychotic medications (Kumari et al., 2002).

Animal models suggest that gonadal hormones can also influence sensorimotor gating. In female rats, PPI varies depending on the phase of the estrus cycle (Koch, 1998) and administration of estrogen to ovariectomized females increases PPI (van den Buuse and Eikelis, 2001). T has also been shown to facilitate PPI in male and female rats (Gogos and van den Buuse, 2003), however, another recent study suggests that in male rats PPI is unaffected by treatment with androgens or estrogens (Turvin et al., 2007). In mice the role of gonadal hormones in PPI has been less studied, and the roles of specific hormone receptors, particularly the AR, in modulating PPI are largely unknown, although studies in aromatase knockout (ArKO) males suggest that estrogenic metabolites of androgen acting through ERs can influence PPI (van den Buuse et al., 2003; Gogos et al., 2006).

One model for exploring the role of the AR in the brain and behavior is testicular feminization mutant (Tfm) mice (Zuloaga et al., 2008). Tfm mice have a mutation in the AR gene that involves a single nucleotide deletion (Charest et al., 1991), which introduces a reading frame shift and premature stop codon, resulting in a shortened transcript and essentially no AR protein (He et al., 1991; Monks et al., 2007). Consequently these mice have virtually *no* sensitivity to androgen through the AR (Drews, 1998). Since this trait is X-linked, only genetic males (XY) are wholly androgen insensitive. To further investigate the role of the AR in anxiety-related behavior, regulation of the HPA axis, and sensorimotor gating in males, we compared behavioral and physiological responses in wt and Tfm male mice. Because Tfm male mice have significantly decreased circulating T levels (Jones et al., 2003) behavior was also assessed in mice provided with exogenous T in adulthood. These studies indicate that the AR regulates the HPA axis and plays a role in many behaviors associated with anxiety in mice, but plays little if any role in regulating PPI.

Method

Animals

C57BL6J mice bred in our Tfm colony at Michigan State University were group housed with a 12/12 L/D cycle, lights on at 0600. The mice in this colony have been sired exclusively by commercially purchased C57BL6J males for over 10 generations. Upon weaning at 21 days old, mice were ear punched and genotyped using a modified polymerase chain reaction (PCR) described elsewhere (Fernandez et al., 2003). Products of this reaction differentiated between the Tfm and the wt allele for the AR, and male versus female, based on the presence or absence of the Sry gene found only on the Y chromosome. All animals received care that meets standards of the National Institutes of Health and all experiments were approved by the MSU Institutional Animal Care and Use Committee.

Procedure

Experiment 1

To address the role of the AR in sensorimotor gating and anxiety-related behavior, 90–120 day old wt male ($N=9$) and Tfm male mice ($N=10$) were tested for PPI and anxiety-related behaviors as described in detail below.

Experiment 2

Since endogenous T levels differ in Tfm and wt male mice (Tfm ≪ wt) and might explain differences in behavior, a second experiment was performed. Wt males were gonadectomized at 70–90 days of age and implanted with Silastic capsules containing T ($N=10$) or nothing (blank, B; $N=9$), while Tfms were sham-operated and also implanted with capsules containing T ($N=10$) or nothing ($N=9$). Tfm males were left gonadally intact because their testes produce negligible amounts of T (Jones et al., 2003; present study). Three weeks later, mice were assessed for sensorimotor gating and anxiety-related behavior as described below.

Experiment 3

To assess whether differences in anxiety-related behavior might be related to differences in HPA axis activation, plasma corticosterone was collected from 90 to 120 day old wt and Tfm male mice at baseline (wt male: $N=10$; Tfm male: $N=10$) or at 20 min following the onset of exposure to an open field with a novel object (wt male: $N=10$; Tfm male: $N=10$).

Experiment 4

To assess whether the time course of HPA axis recovery differed in wt and Tfm males following exposure to an open field with a novel object, 90–120 day old mice were assayed for corticosterone at baseline (wt male: $N=7$; Tfm male: $N=6$) as well as at 20 (wt male: $N=7$; Tfm male: $N=7$), 40 (wt male: $N=6$; Tfm male: $N=7$), 60 (wt male: $N=7$; Tfm male: $N=7$), and 120 (wt male: $N=6$; Tfm male: $N=6$) min after exposure to a novel object in the open field testing arena.

Adult castration and hormone replacement

At 70–90 days, wt male mice were anesthetized with isoflurane and the testes externalized via bilateral incisions made in the scrotum. The vas deferens was tied off with silk suture prior to cutting. For Tfm males, bilateral incisions were made in the perineal cavity in a similar location as the scrotum in wt males. Animals also received a subcutaneous Silastic capsule (1.57 mm inner diameter, 3.18 mm outer diameter: 6 mm effective length) containing either free T (T) or nothing (blank (B)) via a 2 cm incision dorsally at the nape of the neck. These Silastic capsules were designed to deliver normal male physiological levels of T to adult mice. Incisions were closed with wound clips and the analgesic buprenorphine (0.1 mg/kg) was injected sc post operatively.

Behavior testing

Animals in Experiments 1 and 2 were tested for sensorimotor gating (PPI) and anxiety-related behaviors. Testing took place in the following order: PPI, open field/novel object test, elevated plus maze (EPM), and light/dark (LD) box, with each animal receiving a minimum of 72 h between tests. All tests were administered between 1000 and 1400 except for PPI, which was conducted during the dark cycle beginning at 1900. In order to address the possibility that

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exposure to the most fear provoking test (PPI) might alter behavior on the following tests, tests were also conducted in another cohort of intact wt ($n=10$) and Tfm ($n=10$) male mice in order from those judged to be the least to most anxiety provoking (open field/novel object test, light dark box, elevated plus maze, PPI).

Prepulse inhibition

Mice were tested for PPI 1 h after lights off in a room illuminated by dim red light. PPI was measured in acoustic startle response chambers (SR Lab startle response system, San Diego Instruments, San Diego, CA). Animals were placed into the chamber for 18 min, the first 5 min of which is an acclimation period. For the remaining 13 min the fast twitch startle responses of mice were recorded via SR Lab software (San Diego Instruments) to a 100 decibel (dB) tone alone (acoustic startle response), or the same tone preceded by a 100 ms tone of 3, 8, 10, or 15 dB. The prepulse should permit the subject to anticipate the loud pulse, and consequently startle less severely. PPI was calculated as the percentage decrease in startle response relative to responding to the 100 dB tone without a prepulse. Each of these trials was repeated 6 times at pseudo-random intervals. After the test, animals were removed and the chamber was cleaned with 70% ethanol.

Open field/novel object test

Open field/novel object testing for mice took place in a 16″×16″ Plexiglas chamber (Versamax animal activity monitor, Accuscan Instruments Inc, Columbus, OH) that was illuminated from above. The chamber was equipped with a grid of invisible infrared beams, and breaks in beams were used to measure movement characteristics such as rearings (vertical movements that involve removal of the forelimbs from the floor) and anxiety-related indices, including the number of entries into and time spent in the center area of the field or visiting the novel object at the field center. Animals were first placed into a corner of the empty open field and data were collected for 5 min. After 5 min mice were removed, the chamber was cleaned with 70% ethanol, and a novel object (a 2″ d×0.75″ h Petri dish with red and blue tape) was placed in the center of the chamber. Three minutes after the first removal from the open field, the mouse was then replaced into the chamber and data were collected for another 5 min. The chamber was cleaned with 70% ethanol between each test.

Elevated plus maze (EPM)

The EPM (Phenome Technologies Inc.; Lincolnshire, IL) consisted of two open and two closed arms (30×5 cm) that extended from a center platform and was elevated 50 cm above the floor. Testing took place in a dimly lit room with a video camera suspended above the maze to record behavior for assessment at a later time by an observer blind to the experimental condition. During testing, animals were placed in the center area of the EPM facing an open arm and allowed to freely explore for the 10 minute duration of the test. The numbers of entries into and the amount of time spent on the open and closed arms were assessed as indices of anxiety-related behavior. The EPM was cleaned with 70% ethanol between each test.

Light/dark (LD) box

The LD box (Phenome Technologies Inc.; Lincolnshire, IL) consisted of a rectangular box divided into two regions, a dark (21 cm length×24 cm width×19 cm height) and a light (21 cm length×24 cm width×19 cm height) area connected by a (10 cm height×5 cm width) opening between the two. The dark region was constructed of black plastic and was covered by a black plastic lid, while the light region was constructed of clear Plexiglas and was illuminated by a 60 watt light 3 ft directly above it. The small opening connecting the two chambers allowed the animals to freely enter either area. Animals were placed in the light side of the chamber facing the opening to the dark chamber and were allowed to move freely between the two compartments for 10 min with behavior recorded via an overhead video camera. The number of entries into, time spent in the two compartments, and the number of rearings in the light compartment were scored at a later time as indices of anxiety-related behavior by an observer blind to the experimental condition. The chamber was cleaned with 70% ethanol between each test.

Plasma collection, corticosterone and testosterone assay

In Experiment 3, blood was collected from adult mice between 0900 and 1100 at baseline or 20 min after exposure to an open field with a novel object (a 2″ d×0.75″ h Petri dish with red and blue tape). In Experiment 4, blood was collected from adult mice between 0900 and 1100 at 20, 40, 60, and 120 min after exposure to an open field with a novel object or from control mice that remained in their home cage. Exposure to a novel object in an open field was for 10 min, after which they were returned to their home cage where they remained until sacrifice. Control mice remained in their home cage until sacrifice. Mice were deeply anesthetized with isoflurane and decapitated, with trunk blood collected within 2 min of cage disturbance. Blood was also collected from mice in Experiment 2 to verify T levels after supplementation. These mice were deeply anesthetized with sodium pentobarbital and blood was collected through cardiac puncture. All blood was collected in 1.5 ml tubes containing 250 μl of heparin and held on crushed blue ice until centrifugation. Samples were centrifuged at 8 °C for 20 min and plasma was collected and stored frozen at −20 °C. Plasma was assayed for corticosterone and T at the Diagnostic Center for Population and Animal Health at Michigan State University using Coat a Count Corticosterone and Coat a Count Total Testosterone kits (Diagnostics Products Corporation, Los Angeles, CA, USA). All plasma samples were run in duplicate and the results averaged.

Statistical analysis

For PPI, a mixed design ANOVA was used to analyze data for all treatment groups and/or genotypes as between group factors and the different prepulse trials as within group (or repeated measures) factors. Anxiety-related behaviors were analyzed in Experiment 1 using Student's *t*-tests, or in Experiment 2 using a 2×2 analysis of variance (ANOVA) with hormone treatment (T, no T) and genotype (wt male and Tfm male) as independent factors. To compare behavioral differences in mice exposed to the PPI test prior to anxiety tests (test order 1) and mice tested in order from least to most anxiety provoking (test order 2) a 2-way ANOVA was used with genotype and test order (test order 1, test order 2) as independent factors. In Experiment 3, T and corticosterone were analyzed using a 2×2 analysis of variance (ANOVA) using testing condition (open field/novel object exposure, no manipulation) and genotype (wt male and Tfm male) as independent factors. In Experiment 4 differences in corticosterone were analyzed using a two-way ANOVA (genotype×time). All significant main effects or interactions were further analyzed using Newman–Keuls multiple comparisons post hoc tests. Differences were considered significant when $p<0.05$ and all data are reported as means±standard error of the mean (SEM).

Results

Experiment 1

For PPI, a mixed design ANOVA (genotype as a between groups factors and prepulse intensity as a within groups factor) revealed the expected effect of prepulse intensity ($F(3,42)=87.679$, $p<.001$) in which PPI increased as intensity of the prepulse increased. There was also a significant effect of genotype in which wt males showed an overall greater PPI than did Tfm males ($F(1,45)=4.869$, $p<.05$; Fig. 1A). This difference appeared to be independent of prepulse intensity as

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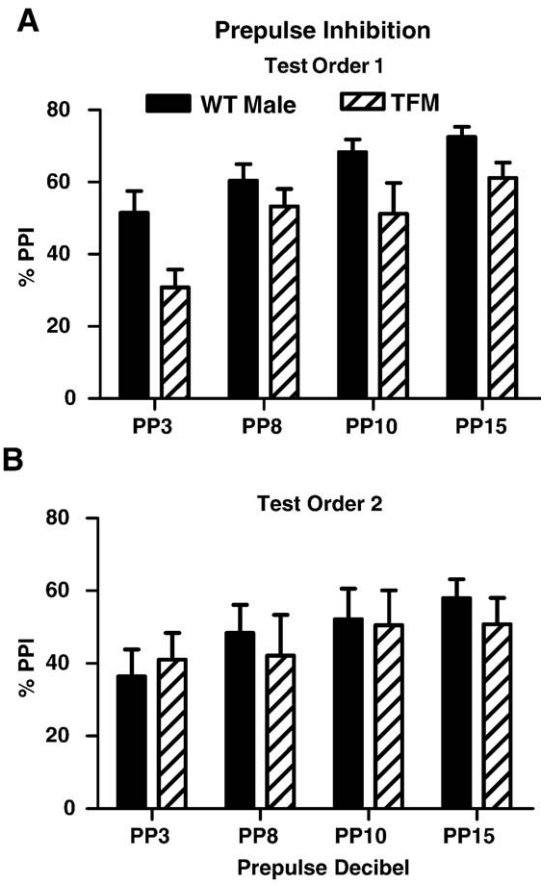

Fig. 1. Prepulse inhibition of the acoustic startle response (PPI) in gonadally intact wt and Tfm male mice exposed to prepulses (PP) of 3, 8, 10, and 15 decibels (dB) 100 ms prior to a loud 100 dB pulse. PPI data are shown in mice from (A) test order 1 (PPI, open field/novel object test, EPM, LD box) and (B) test order 2 (open field/novel object test, LD box, EPM, PPI). PPI increases as the magnitude of the prepulse increases in mice in test order 1 (A) but not in test order 2 (B). * indicates an overall decrease in prepulse inhibition in Tfm compared to wt males ($p<.05$) in test order 1, but there was no significant difference in test order 2. When test orders 1 and 2 are pooled together, no significant difference between Tfm and wt males in PPI is found.

there was only an overall effect of genotype and no significant interaction. No significant difference in startle response to the loud acoustic pulse alone was found in this test (wt males: 63.9±6.2; Tfm males: 50.3±8.4; in AU).

In the open field test, no significant differences were found between wt and Tfm males in the number of entries into or time spent in the center area of the chamber (Fig. 2A). There was also no significant difference in the total number of rearings, although there was a trend in which wt males tended to rear more frequently than did Tfms ($t(17)=1.971$, $p=.065$; Fig. 2E, left-most bars). When mice were exposed to a novel object, independent *t*-tests revealed that wt males entered the center area of the open field containing the object more frequently ($t(17)=2.225$, $p<.05$; Fig. 2B) and spent more time visiting the novel object ($t(17)=2.177$, $p<.05$; Fig. 2B) than did Tfm males. The number of rearings was also greater in wt than Tfm males ($t(17)=3.279$, $p<.01$; Fig. 2E, middle bars).

In the EPM there were no group differences in the number of entries into the open arm of the maze, though wt males did show a trend toward spending more time in the open arms of the maze than

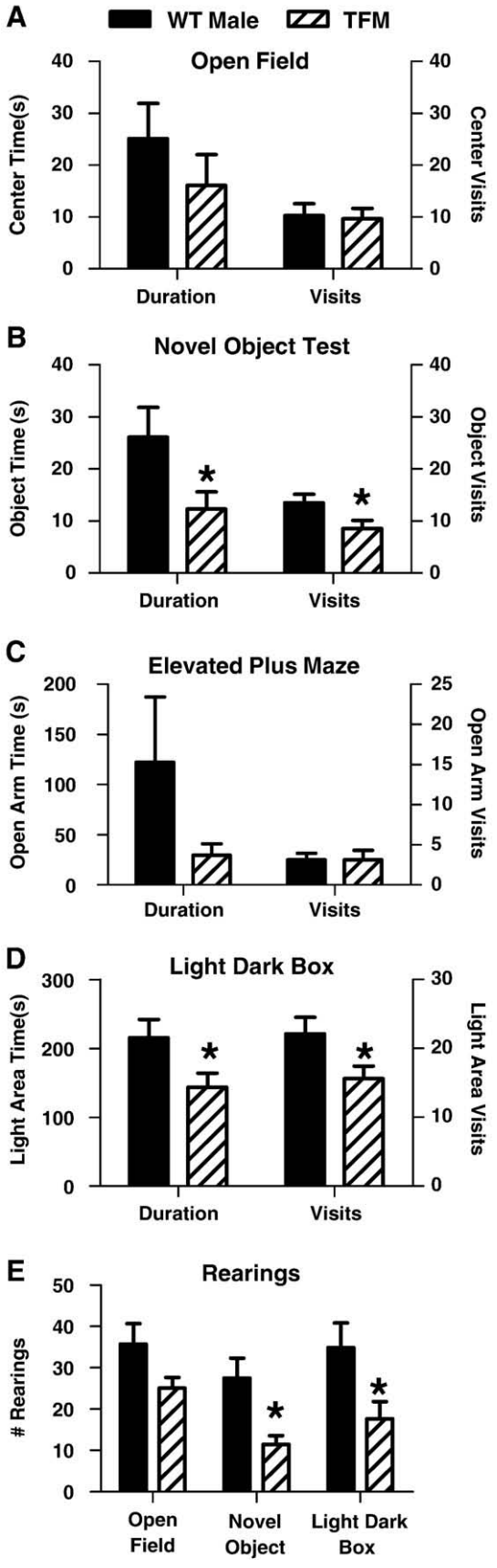

Fig. 2. Anxiety-related behavior in gonadally intact wt and Tfm male mice. The amount of time spent in/with and number of visits to: (A) the center area of the open field, (B) a novel object in the open field arena, (C) the open arms of the elevated plus maze, and (D) the light dark box. (E) depicts the number of rearings in the open field, novel object, and LD box tests. These tests indicate greater anxiety-related behaviors in Tfm mice compared to wt mice in the novel object test and light dark box, but not the other two tests. * indicates $p<.05$ compared to wt males.

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Table 1
Body weight and testosterone levels in wt and Tfm male mice

Mice	Weight	T (nmol/l)
T or B-treated		
WT male (T)	29.38±.60	14.22±2.95
WT male (B)	25.93±.96	2.63±1.52
TFM male (T)	26.49±.56	16.81±2.17
TFM male (B)	25.78±1.38	4.43±2.28
Intact		
WT male	28.38±.70	19.75±4.39
TFM male	24.92±.74	3.26±.52

In T and B-treated mice, body weight was greater in T-treated wt males than all other groups (p's<.05), and T levels were elevated in T compared to B-treated mice (p<.001). In intact untreated mice, wt males had elevated body weights and T levels compared to Tfm males (p<.01). T: testosterone, B: blank.

did Tfm males ($t(17)=1.781$, $p=.09$; Fig. 2C). In the LD box, independent t-tests revealed that wt males showed a greater number of entries into the light area of the box ($t(17)=2.148$, $p<.05$; Fig. 2D), spent more time in the light area ($t(17)=2.178$, $p<.05$; Fig. 2D), and reared more frequently in the light area ($t(17)=2.372$, $p<.05$; Fig. 2E, right-most bars) than did Tfm males.

When behavior tests were conducted in another cohort of wt and Tfm male mice, in order from least to most anxiety-provoking (test order 2), similar group differences and/or similarities were found for all tests except PPI. Time spent visiting the novel object, in the light area of the LD box, and in the open arm of the EPM was greater (or marginally greater) in wt than in Tfm males (($t(18)=3.376$, $p<.01$; ($t(18)=2.290$, $p<.05$); ($t(18)=2.067$, $p=.057$) respectively; data not shown), and again there were no group differences in the open field test. In mice in test order 2 there were no significant main effects of genotype or prepulse intensity for PPI (Fig. 1B) and again acoustic startle responses in the absence of a prepulse did not differ. As a result of the discrepancy in group differences in PPI mice from test orders 1 and 2 were pooled to determine if there was an overall effect of genotype. This analysis did not indicate a significant main effect of genotype for PPI. No significant main effect of test order was found for behaviors in the open field, novel object test, LD box, EPM, or PPI test between mice in test order 1 and test order 2, indicating behaviors in Tfm and wt males were not significantly affected by test order.

Experiment 2

There was a significant main effect of T treatment on body weight with no main effect of genotype or interaction. Specifically, body weight was greater in T-treated wt males than all other groups (all p's<.05; Table 1). Blood concentrations of T also revealed a significant main effect of treatment with no main effect of genotype or interaction. Animals provided with T capsules had circulating T levels that were in the physiological range, slightly lower than in gonadally intact wt males (Table 1). Castrated wt males given B capsules had circulating levels of T that approximated those of untreated Tfm mice in both studies (Table 1).

A mixed design ANOVA using genotype and hormone treatment as between groups factors and prepulse intensity as a within groups factor again revealed the expected effect of prepulse intensity ($F(3,96)=48.293$, $p<.001$; Fig. 3B) in which PPI increased as the intensity of the prepulse increased. However, no significant main effects or interactions were found between groups for PPI in this cohort. As in Experiment 1, there were no significant group differences for startle response to the 100 dB pulse alone (Fig. 3A).

In the open field test, a 2×2 ANOVA revealed no significant main effects or interactions between groups in the number of entries into and time spent in the center area of the chamber (Fig. 4A), or in the number of rearings (Fig. 4E, left-most bars). In the novel object test there was a significant main effect of genotype ($F(1,34)=9.35$, $p<.05$), with no main effect of treatment or interaction, indicating that as a group Tfm males spent less time visiting the novel object than did wt males, replicating the results of Experiment 1 in gonadally intact mice. Post hoc comparisons revealed significant differences between T-treated wt males and all other groups (p's<.05; Fig. 4B, left), indicating that T increases this measure, but only in wt males and not in Tfm males. A significant main effect of genotype was also revealed for the number of rearings in the novel object test ($F(1,34)=6.714$, $p<.05$), with no main effect of treatment or interaction, indicating that wt males reared more than did Tfms. Post hoc comparisons revealed significant differences between T-treated wt males and both Tfm groups (T or no T, p's<.05; Fig. 4E, middle bars). No significant main effects or interactions were found for the number of novel object visits, though there was a trend toward a main effect of treatment ($F(1,34)=3.323$, $p=.076$; Fig. 4B, right).

In the EPM no significant main effects or interactions were found for time spent on or number of visits to the open arms (Fig. 4C). In the LD box a significant main effect of treatment was found for time spent in the light area ($F(1,34)=4.209$, $p<.05$), with no significant main effect for genotype or interaction. Post hoc tests revealed that T-treated wt males spent more time in the light area than did either B-treated wt males or T-treated Tfm males (p's<.05; Fig. 4D, left), though T-treated wt males did not significantly differ from B-treated Tfm males. A significant main effect of genotype was found for the number of rearings in the light area of the LD box ($F(1,34)=6.356$, $p<.05$), with no significant main effect of treatment or interaction. Post hoc comparisons revealed significant differences between T-treated wt males and all other groups (p's<.05; Fig. 4E, right bars), indicating that T-treated wt males reared more than any other group in the light area of the LD box. No significant main effects or inter-

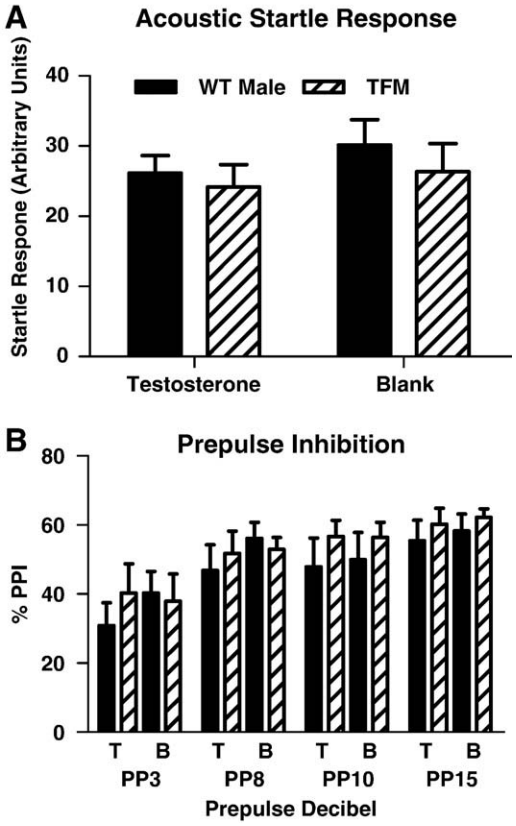

Fig. 3. (A) Acoustic startle response and (B) prepulse inhibition in T and B-treated wt and Tfm male mice. No significant group differences were found in acoustic startle response or prepulse inhibition. T: testosterone, B: blank.

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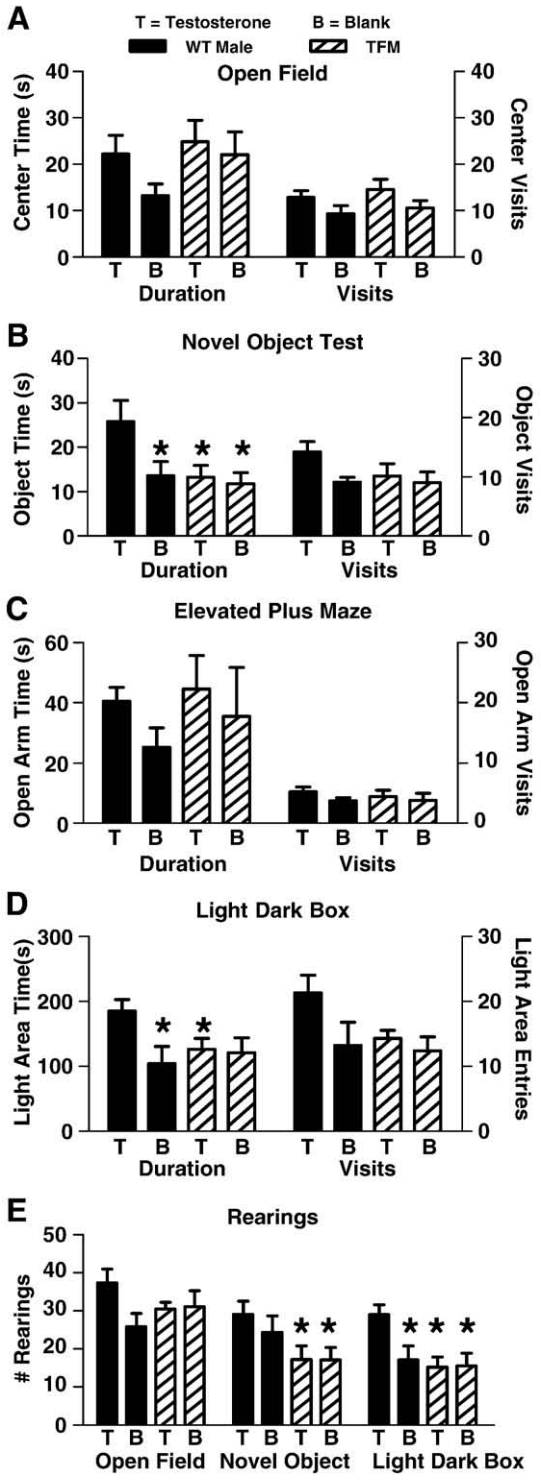

Fig. 4. Anxiety-related behavior in T and B-treated wt and Tfm male mice. The amount of time spent in/with and number of visits to: (A) the center area of the open field, (B) a novel object, (C) the open arms of the elevated plus maze, and (D) the light area of the light dark box. (E) depicts the number of rearings in the open field, novel object, and LD box tests. These results confirmed the previously found differences between wt males and Tfm males for the novel object and LD box tests (see Fig. 2) and suggest that T amelioration of anxiety-related behaviors are mediated by AR, since Tfm animals showed no behavioral response to T treatment in any test. * indicates $p<.05$ compared to T-treated wt males. T: testosterone, B: blank.

actions were found for the number of light area visits, although there was a trend toward a main effect of treatment ($F(1,34)=3.966$, $p=.054$; Fig. 4D, right).

Experiment 3

Two-way ANOVA revealed a significant main effect of exposure ($F(1,38)=908$, $p<.001$) in which mice exposed to an open field with a novel object showed an increased corticosterone response 20 min after exposure compared to mice in the basal condition (left in their cage). There was also a significant main effect of genotype ($F(1,38)=177.4$, $p<.001$) with Tfm males showing an increased corticosterone response compared to wt males. Post hoc comparisons revealed that Tfm males had greater blood corticosterone levels at baseline ($p<.01$) and after exposure to an open field with a novel object ($p<.001$). A significant interaction was also found between exposure and genotype on corticosterone concentrations ($F(3,36)=69.1$, $p<.001$), which indicated that the increase in corticosterone from baseline to that induced by exposure to a novel object was greater in Tfm males than in wt males (Fig. 5A). Two-way ANOVA revealed the expected significant main effect of genotype in T levels ($F(1,38)=134$, $p<.001$), with no main effect of exposure or interaction between genotype and exposure. Specifically, compared to wt males, Tfm males had lower levels of T (Table 1). As expected, body weight was also significantly greater in wt than in Tfm males ($t(17)=3.382$, $p<.01$; Table 1).

Experiment 4

A 2-way ANOVA confirmed the expected main effect of time on corticosterone levels ($F(4,52)=55.58$, $p<.001$) indicating that compared to basal corticosterone levels, Tfm and wt males had greater corticosterone levels overall after exposure to a novel object. A main effect of genotype was also revealed in which Tfm males showed overall greater blood corticosterone concentrations compared to wt

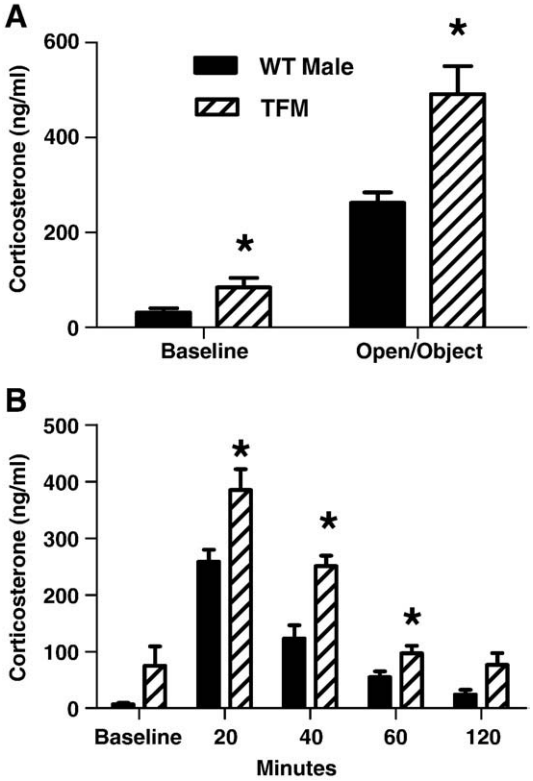

Fig. 5. (A) Plasma corticosterone levels at baseline and 20 min after initial exposure to an open field with a novel object were elevated in Tfm males compared to wt males. * indicates $p<.01$ compared to wt males. (B) Plasma corticosterone levels at baseline and 20, 40, 60, and 120 min after initial exposure to an open field with a novel object in Tfm and wt males. * indicates $p<.05$ compared to the same time point in wt males.

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males ($F(1,55)=33.67$, $p<.001$). Post hoc comparisons revealed that Tfms had higher corticosterone concentrations compared to wt males at 20, 40, and 60 min (p's$<.05$; Fig. 5B).

Discussion

Tfm male mice showed increased indices of anxiety in several behavioral tests (novel object and LD box tests), but not others (open field and EPM), indicating a task-specific increase in anxiety-related behavior in Tfm males compared to wt males. In the novel object and LD box tests, Tfm males showed decreased exploration of a novel object and of the light area of the LD box as well as a generalized decrease in exploratory behavior in these tests, as assessed by the number of rearings. T treatment in gonadectomized wt males decreased indices of anxiety in the novel object and LD box tests suggesting that T treatment in adult male mice can produce anxiolysis. However, these behaviors were unaffected by T treatment in Tfm males, which indicates that T's anxiolytic action in wt males is likely mediated through the AR. These findings support previous research indicating that the AR is involved in the regulation of anxiety-related behavior in rodents (Edinger and Frye, 2006; Fernández-Guasti and Martínez-Mota, 2005; Rizk et al, 2005). Why behaviors differed between Tfm and wt males in some tests of anxiety and not others is not readily apparent as these tests all fall within the broad category of exploration based anxiety tests. These results suggest a moderating influence of AR on anxiety-related behaviors in male mice, which variables specific to the novel object and LD box tests revealed.

Since the Tfm mutation is present from ontogeny, it is unclear whether differences between wt and Tfm males in anxiety-related behavior are the result of "organizational" or "activational" influences of ARs (Arnold and Breedlove, 1985). Other findings in rodents indicate that activation of ARs in adulthood plays a prominent role in regulating anxiety-related behavior (Edinger and Frye, 2006; Fernández-Guasti and Martínez-Mota, 2005). In our study, decreased anxiety-related behavior in wt males given T capsules versus B capsules confirms that T can act in adulthood to affect this behavior, although it does not directly implicate the AR. It is possible that decreased AR activation during development in Tfm males resulted in their increased indices of anxiety. Little is known about the organizational role of ARs in anxiety, although a study showed that neonatal treatment of rats with the AR antagonist flutamide did not affect indices of anxiety in the elevated plus maze (Zimmerberg and Farley, 1993).

Corticosterone levels were also elevated in Tfm males compared to wt males at both baseline and 20 min after initial exposure to an open field with a novel object (Experiment 3). During recovery, the corticosterone response remains consistently elevated in Tfm males compared to wt males at 40 min and 60 min (Experiment 4). These findings indicate that the increased anxiety-related behavior in Tfm male mice may be related to a hyper-activation of the HPA axis. Previous studies suggested a relationship between trait anxiety and increased HPA axis activity after exposure to rodent tests of anxiety. Rats bred for high compared to low anxiety behavior show elevations in stress hormones ACTH and corticosterone that correlate with increased indices of anxiety in the EPM and open field (Landgraf et al., 1999; Salomé et al., 2004).

Androgens have been shown to play a role in the regulation of the HPA axis response to stress in other models. Gonadectomy of adult wt male rats increased the release of corticosterone and ACTH following footshock stress or exposure to an open field (Handa et al., 1994). Furthermore, T or DHT replacement in gonadectomized rats decreased the rise in stress hormones to levels similar to those of intact rats (Handa et al., 1994). Androgen's influence on the HPA axis does not appear to be solely activational, however. Neonatal gonadectomy in male rats increased the corticosterone response to restraint stress, an increase that is not reduced by adult testosterone propionate (TP) replacement as it is in rats gonadectomized as adults (McCormick et al., 1998). Neonatal androgen exposure can also alter the female corticosterone response: female rats administered TP on the day of birth showed decreased basal and stress induced corticosterone levels (Seale et al., 2005a). Other evidence supports the notion that the AR is involved in the organization of the HPA axis (McCormick and Mahoney, 1999; Seale et al., 2005b). Seale et al. (2005b) demonstrated that perinatal AR blockade, by administration of the AR antagonist flutamide, increased both basal and stress induced corticosterone levels in adulthood compared to rats given vehicle perinatally. Furthermore, serum corticosterone and ACTH levels are elevated in AR knockout male mice, in which AR deficiency is present from ontogeny (Miyamoto et al., 2007), as in Tfm males.

Previous research therefore indicates that increased corticosterone levels found in Tfm male mice could be related to organizational influences, activational influences, or both, resulting from a lack of functional ARs. However, since Tfm males have decreased levels of T compared to wt males, it is also possible that when provided with ample T, Tfm males would show corticosterone levels similar to wt males. Evidence suggests that T, after metabolism to DHT, can be converted to 3β-diol which reduces HPA axis activity via activation of ERβ (Lund et al., 2004; Lund et al., 2005; Lund et al., 2006). Therefore, without additional experiments, we cannot conclude that the increased corticosterone levels seen in Tfm male mice result directly from a lack of functional ARs. However, Tfm male rats, which have elevated T levels compared to wt males, also show an increased corticosterone response compared to wt males 20 min following exposure to an open field with a novel object (Zuloaga et al., submitted for publication). Therefore, even when provided with similar T, Tfm male mice may still continue to show increases in corticosterone compared to wt males. This outcome would be consistent with our behavioral measures of anxiety suggesting that Tfm male mice show increased anxiogenesis regardless of endogenous T levels.

Indirect evidence suggests that in Tfm male mice, T levels are near the normal male range during the perinatal period (Goldstein and Wilson, 1972) and decrease during development largely due to a reduction in the testicular enzyme 17α-hydroxylase (Murphy and O'Shaughnessy, 1991). Thus, Tfm male mice may have ample T substrate to activate ERs perinatally, leading us to suspect that the increased HPA axis activity seen in Tfm male mice may indeed reflect a lack of functional ARs, although when AR is critical remains unclear.

Since plasma corticosterone binding globulin (CBG) levels were not measured in this study, it is possible that CBG concentrations are elevated in Tfm males and could contribute to differences in corticosterone levels. An increase in CBG levels would indicate that more corticosterone would need to be released in order for similar corticosterone receptor binding to occur, because more circulating corticosterone is being bound by CBGs and is therefore rendered biologically inactive. Increased T has been shown to decrease plasma CBG concentrations (Viau and Meaney, 2004). Therefore, if indeed a decrease in circulating T (or a decrease in functional ARs independent of circulating T) in Tfm males increases plasma CBGs, elevated plasma corticosterone levels in Tfm males may reflect an attempt to achieve normal receptor activation.

Intact Tfm male mice also showed an overall decrease in PPI compared to wt male mice in test order 1, suggesting that the AR may be involved in sensorimotor gating. However, in test order 2 there were no group differences in PPI and in Experiment 2 neither T treatment nor genotype affected PPI. These findings suggest that the original differences found between wt and Tfm male mice may be subtle and somewhat unreliable. Alternatively, group differences in PPI may have been reduced by prior exposure to tests of anxiety in intact animals. However, hormone manipulated mice in Experiment 2 were tested for PPI without prior exposure to tests of anxiety and did not show differences in PPI, indicating that test order did not contribute to group differences. Together these data suggest that in mice

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the role of T and ARs in the regulation of PPI is minimal. This corresponds with a report of normal PPI in mice with low circulating T levels (Umehara et al., 2006).

Tfm males provide a useful model for examining the role of ARs in behavioral and physiological responses, while avoiding some of the limitations of pharamacological AR agonists (e.g., DHT) and antagonists (e.g., flutamide). DHT, along with activating ARs, can be metabolized to 3α-androstanediol (3α-diol) which has a low affinity for ARs but a high affinity for GABA receptors. 3α-diol also appears to play a role in anxiolysis in rodents, as its administration decreases anxiety-related behavior (Frye and Edinger, 2004; Edinger and Frye, 2004; Edinger and Frye, 2005). An estrogenic metabolite, 3β-diol, which binds ERs with greater affinity for ERβ than ERα, can also be derived from DHT (Kuiper et al., 1998; Lund et al., 2004). 3β-diol administration has also been demonstrated to reduce anxiety-related behavior and activation of the HPA axis through its actions on ERβ (Lund et al., 2004; Lund et al., 2005; Lund et al., 2006). Thus effects of DHT treatment on anxiety-related behaviors and HPA axis activity could be mediated either through AR, ERβ, or GABA receptors.

A limitation of the Tfm model is that Tfm male rats have been reported to show decreased aromatase activity in several areas of the brain (Roselli et al., 1987). Therefore it is possible that T treatment in Tfm males failed to affect anxiety-related behavior because there was insufficient ER activation due to a decrease in the conversion of T to estrogens. However, Tfm male mice have been reported to show both similar and slightly decreased levels of aromatase activity in the hypothalamus compared to wt males (Naftolin et al., 1975; Rosenfeld et al., 1977). In the study that indicated a decrease in Tfm aromatase activity, it was the conversion of T to the less potent estrogen, estrone, which contributed to an overall decrease in aromatization, while the conversion of T to E2 was actually elevated in Tfm male mice (Rosenfeld et al., 1977). Estrone, unlike E2, has a much higher affinity for ERα than ERβ, and evidence suggests that a decrease in ERα activation would not contribute to increased anxiogenesis (Lund et al., 2005; Krezel et al., 2001). Furthermore, aromatase knockout (ArKO) male mice have been shown to exhibit normal levels of anxiety behavior (Dalla et al., 2005), suggesting that aromatization of T to estrogens plays a minimal role in the display of these behaviors in male mice.

In conclusion, the present findings in Tfm mice indicate that the AR is involved in the regulation of anxiety-related behaviors in males, as demonstrated by differences between wt and Tfm males in several tests of anxiety. Furthermore, these differences in anxiety-related behavior may be related to an increased activation of the HPA axis in Tfm males since they show increased levels of blood corticosterone at baseline as well as at several time points following exposure to an open field with a novel object. Further work will be needed to explore whether ARs act during development and/or adulthood to affect anxiety-related behaviors. On the other hand, the role of the AR in sensorimotor gating in mice, as indicated by PPI, appears minimal, suggesting that any role of gonadal steroids on sensorimotor gating may be mediated via estrogen receptors rather than ARs.

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